

Developmental Changes in Neural Corticosteroid Receptor Binding Capacity in Altricial Nestlings

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ABSTRACT: Altricial nestlings typically do not show an adrenocortical response during the early post-hatch period. This may be a result of an immature hypothalamic-pituitary-adrenal axis, or an enhanced control of the axis by negative feedback. To examine whether the dampened adrenocortical response is due to higher receptor densities in hypothalamus and hippocampus, the major sites for negative feedback and tonic inhibition, we explored the ontogenetic changes in glucocorticoid (GR) and mineralocorticoid receptor (MR) binding capacities in the brain of white-crowned sparrow nestlings. During the 10-day nes-

ting period, MR binding capacity decreased with age, whereas GR capacity was not affected. In addition, this overall decline in MR levels was driven entirely by a decline in cerebellar MR. No age-related changes were observed in hippocampal or hypothalamic areas. Our findings suggest that enhanced negative feedback does not play a major role in the attenuated adrenocortical responses seen in white-crowned sparrow nestlings. © 2010 Wiley Periodicals, Inc. *Develop Neurobiol* 70: 853–861, 2010

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INTRODUCTION

Several mammalian and fish species exhibit a period of hyporesponsiveness to certain stressors during critical developmental phases, called a stress hyporesponsive period, SHRP (Sapolsky and Meaney, 1986; Vazquez, 1998; Walker et al., 2001). This is characterized by attenuated secretions of corticotrophin and glucocorticoids from the pituitary and adrenal/interrenals. Unfavorable effects of glucocorticoids on growth (Mashaly, 1991; Morici et al., 1997; Glennemeier and

Denver, 2002; Spencer et al., 2003; Hayward and Wingfield, 2004; Meylan and Clobert, 2005; Saino et al., 2005; Wan et al., 2005; Janczak et al., 2006; Wada and Breuner, 2008), immune function (Morici et al., 1997; Rubolini et al., 2005), neurogenesis (McEwen, 1987; Kanagawa et al., 2006; Heine and Rowitch, 2009), and survival (Mashaly, 1991; Saino et al., 2005; Janczak et al., 2006) led researchers to believe that the SHRP is an adaptive characteristic, serving to minimize detrimental effects of glucocorticoids during development.

In birds, little work has been done on a possible SHRP, especially in altricial species which are morphologically and behaviorally immature at hatching. Consequently, it is expected that glucocorticoid secretion would be limited in newly hatched altricial birds. In fact, altricial young have a limited response to handling stress during the early post-hatch period (Romero et al., 1998; Sims and Holberton, 2000; Wada et al., 2007, 2009a,b). This low corticosterone (CORT) secretion early in development may be the result of an immature hypothalamic-pituitary-adrenal

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(HPA) axis or an enhanced negative feedback control on the axis.

Negative feedback on the HPA axis controls the adrenocortical response to stress via corticosteroid receptors in hypothalamus and hippocampus (i.e., Sapolsky et al., 1984; Herman et al., 1989; Sapolsky, 1991). There are two types of corticosteroid receptor: high affinity, mineralocorticoid receptors (MR, Type I) and low affinity, glucocorticoid receptors (GR, Type II), (Breuner and Orchinik, 2009). In the hippocampus and hypothalamus, MR is occupied under basal conditions and is thought to mediate tonic inhibition during the diurnal phase (Reul and De Kloet, 1985; Dallman et al., 1987; De Kloet and Reul, 1987), while GR is occupied during a stress response and promotes negative feedback on elevated CORT (Reul and De Kloet, 1985). In addition, these two receptor types differ in their distribution within the brain. In mammals, MR is primarily found in the hippocampus (Pryce, 2008). On the other hand, GR is widely spread throughout the brain with high density in cerebral cortex, hippocampus, thalamus, and hypothalamus (Diaz et al., 1998; Pryce, 2008). These are also age-specific patterns in receptor densities; GR is most abundant in hippocampus in adults, while neonates have the highest number in cerebellum (Pavlik and Buresova, 1984).

Neural GR and MR may be involved in regulation of the SHRP. In rats (*Rattus norvegicus*), there is no strong support that the SHRP is controlled by hippocampal or hypothalamic GR and MR (Pryce, 2008). However in guinea pigs (*Cavia porcellus*), hippocampal MR mRNA and hypothalamic GR mRNA decrease in late gestation concomitant to a rise in plasma cortisol levels (Owen and Matthews, 2003). This suggests that the rise in cortisol in late gestation is due to a release of tonic inhibition and reduction of negative feedback to the HPA axis. In birds, very little is known about the ontogenetic change in GR and MR distribution and the regulation of the HPA axis during development. One study on chicken embryos, however, showed that negative feedback through corticosteroid receptors is functional between embryonic days 15 and 18, several days before the surge in CORT at hatching (Bordone et al., 1997). Thus it is possible that the dampened adrenocortical responses to handling seen in early-stage altricial nestlings are due to an enhanced control of the HPA axis by corticosteroid receptors.

In this study, we investigated neural GR and MR binding capacities during nestling development in white-crowned sparrows (*Zonotrichia leucophrys nuttalli*). We hypothesized that the dampened adrenocortical response observed in this species during the

early nestling period (Wada et al., 2007) may be due to higher receptor densities in hypothalamus and hippocampus, i.e., enhanced negative feedback and tonic inhibition. Using cytosol binding assays, we first explored the general ontogenetic change in GR and MR levels in the whole brain, then examined the age-specific changes in five discrete regions of the brain.

MATERIALS AND METHODS

Animals

Nuttall's white-crowned sparrow nestlings were obtained from nests at the Bodega Marine Reserve of University of California, Davis. Experiment 1 and 2 were conducted between April and June of 2004 and in May and June of 2006, respectively. To determine the nestlings' age, nests were checked every 2–3 days to note the date of hatching, or various physical characteristics of nestlings were compared with those of known aged nestlings (Wada and Breuner, 2008). The nestling period (~10 days) was divided into three age groups for these experiments: days (D) 1–3, 4–6, and 7–9. White-crowned sparrow nestlings hatch with minimal down and closed eyes (Banks, 1959). Nestling growth rate is at its highest during D1–3 (Banks, 1959), and eyes start to open near the end of this period (personal observation). During D4–6, nestlings attain thermoregulatory ability and coordination of movement. During the last stage (D7–9), nestlings are more alert, can exhibit threat displays, and may fledge if disturbed. In this last stage, energy is allocated away from gaining mass and toward growing feathers and maintaining body temperature (Banks, 1959).

Excessive binding of endogenous CORT to corticosteroid receptors at the time of sacrifice can lead to underestimation of receptor levels. Thus, we inhibited stress-induced CORT secretion with mitotane (ortho, para, dichlorodiphenyl dichloroethane), (Breuner et al., 2003; Breuner and Orchinik, 2009). Mitotane is a pharmacological agent that selectively destroys glucocorticoid-producing cells in adrenals (Martz and Straw, 1977; Maher et al., 1992; Hahner and Fassnacht, 2005). A single injection of mitotane is shown to eliminate adrenocortical response within 36 h in sparrows (Breuner et al., 2000). To the best of our knowledge, there is no study indicating that a short-term exposure to mitotane affects GR and MR densities and distributions. It is possible however, that adrenalectomy can alter GR and/or MR levels. For example, Reul et al. (1987) found a slight increase in GR, but not MR, within 24 h of adrenalectomy in rats. On the other hand, Kalman and Spencer (2002) demonstrated that MR protein levels could be upregulated within 12 h of adrenalectomy, but GR protein levels did not increase until 24-h post-adrenalectomy. Although we do not know whether mitotane increases GR and/or MR levels within 24–36 h, pharmacological adrenalectomy with mitotane, especially when all individuals are treated

equally, allows as accurate an estimation of corticosteroid receptors as we can provide. Twenty-four to thirty-six hours prior to perfusion, each nestling was weighed, and the appropriate volume of mitotane ($300 \text{ mg mitotane mL}^{-1}$ peanut oil, 1.2 g kg^{-1} bird) was injected into the abdominal cavity or the pectoralis muscle. The nestlings were then returned to their nests until the next day. On the day of perfusion, birds were captured from their nests and the initial blood sample for baseline CORT was collected from the alar vein within 4 min of capture (Wada et al., 2007). They were then transported to the laboratory in a nest covered with an opaque cloth. Immediately after collecting the second blood sample (preperfusion), birds were anesthetized with Nembutal (Sodium Pentobarbital, 50 mg mL^{-1} , $\sim 0.1 \text{ g kg}^{-1}$ bird). They were then perfused for ~ 5 min with 0.75% avian saline with heparin ($1000 \text{ USP U L}^{-1}$).

Immediately after perfusion, brains were removed and frozen at $\leq -40^\circ\text{C}$ until the assay. The following steps were taken for each brain. In Experiment 1, the brain was analyzed as a whole; the brain was divided longitudinally, and each half was analyzed in a separate assay to obtain two estimates of specific bindings from each nestling. The results from each assay were averaged for the data analysis. In Experiment 2, five regions of the brain (optic lobe, cerebellum, hindbrain, diencephalon, and hippocampus/HVC) were dissected on ice immediately after perfusion using a scalpel and scissors, following the protocol described in Soma et al. (1999). After the cranium was removed, the cerebellum was removed and frozen on dry ice. Next, the hippocampus and HVC area was collected by cutting away a trapezoidal piece of $\sim 1\text{-mm}$ depth along both sides of the midline and posterior to the midway in the rostral-caudal axis, angled toward the midline so that the caudal-most side was $\sim 1\text{-mm}$ long. The rest of the brain was then placed on wet ice with its ventral side facing up. The optic lobes were cut away with a pair of small scissors. The entire hindbrain was collected by cutting at the level of mammillary bodies. The diencephalon was separated from telencephalon and collected into separate tubes. These five regions were frozen separately on dry ice and analyzed in five separate assays.

Cytosol Preparation

Individual receptor levels for both experiments were determined using point-sample assays, following Breuner and Orchinik (2001). Affinities of CORT to GR and MR at different ages were determined using equilibrium saturation binding assay. All assay parameters (time of incubation, temperature, and protein concentration) were optimized for white-crowned sparrow nestling brain tissue. In all assays, brain tissue was first homogenized in TEGMD buffer (10 mM Tris , 1 mM EDTA , 10% glycerol, $20 \text{ mM molybdic acid}$, and $5 \text{ mM dithiothreitol}$, pH 7.4) with a glass homogenizer and vortexed with an equal volume of dextran-coated charcoal (1% charcoal, 0.1% Dextran in TEGM). The samples were then centrifuged for 1 h at 4°C at $104,000g$. Supernatants of each sample were used in the cytosolic receptor assays.

Cytosolic Receptor Assay

Prepared cytosol was incubated with equal volumes of $\sim 15 \text{ nM } ^3\text{H-CORT}$ and either TEGM buffer (GR and MR total binding), 100 nM cold RU486 in TEGM (MR total binding), or $1 \text{ }\mu\text{M}$ cold CORT in TEGM (nonspecific binding) for 3 h at room temperature. The volume of each solution was $50 \text{ }\mu\text{L}$ in Experiment 1 and the equilibrium saturation binding assay, and $25 \text{ }\mu\text{L}$ in Experiment 2; volumes were reduced in the latter experiment due to constraints of tissue volume. After the incubation period, samples were filtered and rinsed through a Brandel harvester with 9 mL TEM buffer (5 mM Tris , 1 mM EDTA , $10 \text{ mM molybdic acid}$, pH 7.4) to capture the $^3\text{H-CORT}$ -receptor complex on a GF/B filter. Filters used in the assay were soaked in 3% PEI in TEM buffer for 1 h at 4°C prior to harvesting. To quantify the protein concentrations in each tissue sample, the remaining cytosol was incubated with $200 \text{ }\mu\text{L}$ of Bradford reagent and read against a standard curve at 595 nm with a Multiskan Ascent microplate reader.

Mineralocorticoid and glucocorticoid receptor capacities were estimated for each individual after the assay. Because RU486 saturates GR, MR specific binding was calculated as MR total binding minus nonspecific binding. Glucocorticoid receptor capacities were calculated as GR and MR total binding minus nonspecific binding and MR specific binding. Then the values were corrected for the protein concentrations in each sample to obtain fmol/mg protein. When nonspecific binding was larger than total binding, or MR total binding was higher than total binding, they resulted in negative values. These were converted to 0 for the data analyses.

Sex Determination

The DNA extraction and PCR protocols for sex determination were modified after Freeman-Gallant et al. (2001). On a shaker, red blood cell samples were incubated with Tris-EDTA (TE) buffer, 20% SDS, and proteinase K at 65°C for 2 h. DNA was extracted using a phenol, phenol-chloroform mixture, and chloroform in three separate steps; DNA-reagent mixture was centrifuged for 10 min at $12,300g$ after each step. Ammonium acetate and 100% ethanol was added to the supernatant of the last extraction step to precipitate the DNA. DNA was purified using 70% ethanol, then TE buffer was added to re-suspend the DNA. The DNA samples were amplified in a PCR machine with forward (GAGAACTGTGCAAAACAG) and reverse primers (TCCAGAATATCTTCTGCTCC). Amplified DNA samples were run through an ethidium bromide stained agarose gel and read over a UV light. Samples of adult DNA with known sex were run next to the nestling samples to confirm the sexing results.

Statistical Analysis

Most analyses were completed with JMP 5.0.1 (Cary, NC); repeated measures ANOVA were performed in SPSS 12.0.

Table 1 Affinities (K_d) of Corticosteroid Receptors in White-Crowned Sparrow (*Zonotrichia leucophrys nuttalli*) Nestling Brain at Different Ages

Age	Ligand	Preferred Model	K_d (nM) \pm SE	
			K_d 1	K_d 2
Days 1–3	³ H CORT	2 site	0.229 \pm 0.180	14.39 \pm 16.08
	³ H CORT+RU486	1 site	0.199 \pm 0.033	
Days 4–6	³ H CORT	1 site	0.398 \pm 0.106	
	³ H CORT+RU486	1 site	0.041 \pm 0.021	
Day 7	³ H CORT	1 site	1.414 \pm 0.303	
	³ H CORT+RU486	1 site	0.758 \pm 0.221	

Data shown are K_d s of MR and GR combined (tissue incubated with ³H CORT) and MR only (tissue incubated with ³H CORT and 100 nM RU486).

Effects of mitotane on plasma CORT levels were analyzed using repeated measures ANOVA. Equilibrium saturation binding curves were drawn and K_d s were determined using GraphPad Prism 4 (San Diego, CA). Prism determines which model best fit the data, resulting in support for a one-site model (the data are best fit by a model with only one binding site for CORT) or a two-site model (the data are best fit by a model where there are two separate binding sites for CORT). The effect of age on K_d was determined by one-way ANOVA with Tukey HSD. For the point sample receptor data, homogeneity of variances was tested using Levene's test; when it resulted in p value of 0.05 or lower, data were transformed to fourth root. For Experiment 1, sex difference was determined using t -test due to a lower sample size and missing sex data on some individuals. Since there was no difference between the sexes, they were combined and analyzed for the effect of age using one-way ANOVA with Tukey HSD. For Experiment 2, we used ANCOVA with age as a main factor and sex as a covariate. For the brain growth data, the effect of age was determined using one-way ANOVA followed by Tukey HSD. Data points deviating two standard deviations away from the mean were classified as outliers and excluded from the analysis (1 out of total 396 data points). Data are presented as mean \pm SE. When $p \leq 0.05$, the null hypothesis was rejected.

RESULTS

Affinities of GR and MR

The equilibrium saturation binding assay showed that GR for D1–3, D4–6, and D7 was best fit by two-site, one-site, and one-site model, respectively (Table 1 and Fig. 1). However, the preference for two-site model over one-site model for D1–3 was likely caused by outliers since GR was best fit by one-site model when ³H CORT concentrations were extended to 12 nM (data not shown). MR for all age groups was best fit by a one-site model. When MR and GR of all ages were analyzed together, receptor affinities differed significantly ($F = 5.85$, $p = 0.0002$). How-

ever when the ontogenetic change in MR and GR affinities were examined, K_d of neither receptors changed with age (post-hoc analysis, $p > 0.05$).

Experiment 1: Ontogenetic Change in GR and MR Binding Capacities in the Whole Brain

Plasma CORT rose slightly but significantly in the 91 \pm 1.6 min from capture to perfusion (time: $F = 6.23$, $p = 0.025$; age: $F = 0.36$, $p = 0.701$; age \times time: $F = 1.51$, $p = 0.25$). Post-hoc analysis indicated that mitotane treatment successfully suppressed CORT levels in the first two age groups (age \times time for D1–3 and D4–6: $p > 0.29$). The oldest age group retained a small but significant increase in CORT prior to perfusion (age \times time for D7: $p = 0.015$; Fig. 2). However, there was no correlation between preperfusion CORT levels and MR binding capacities ($r^2 = 0.003$), and no age difference in preperfusion levels of CORT ($p > 0.60$).

There was no sex difference in GR or MR capacities ($F = 0.06$, $p = 0.812$; $F = 0.07$, $p = 0.797$, respectively). When both sexes were analyzed together, GR binding capacity did not change significantly with age ($F = 0.36$, $p = 0.705$) while MR capacity decreased with age ($F = 3.69$, $p = 0.044$), (Fig. 3). Pairwise comparisons showed that D1–3 nestlings had significantly higher MR capacity than D7 nestlings ($p \leq 0.05$).

Experiment 2: Ontogenetic Change in GR and MR Binding Capacities in Five Regions of the Brain

Overall, age did not affect GR or MR binding capacities in optic lobe, hindbrain, diencephalon, or hippocampus/HVC, or GR capacity in cerebellum (Table 2). However, MR capacity in cerebellum decreased

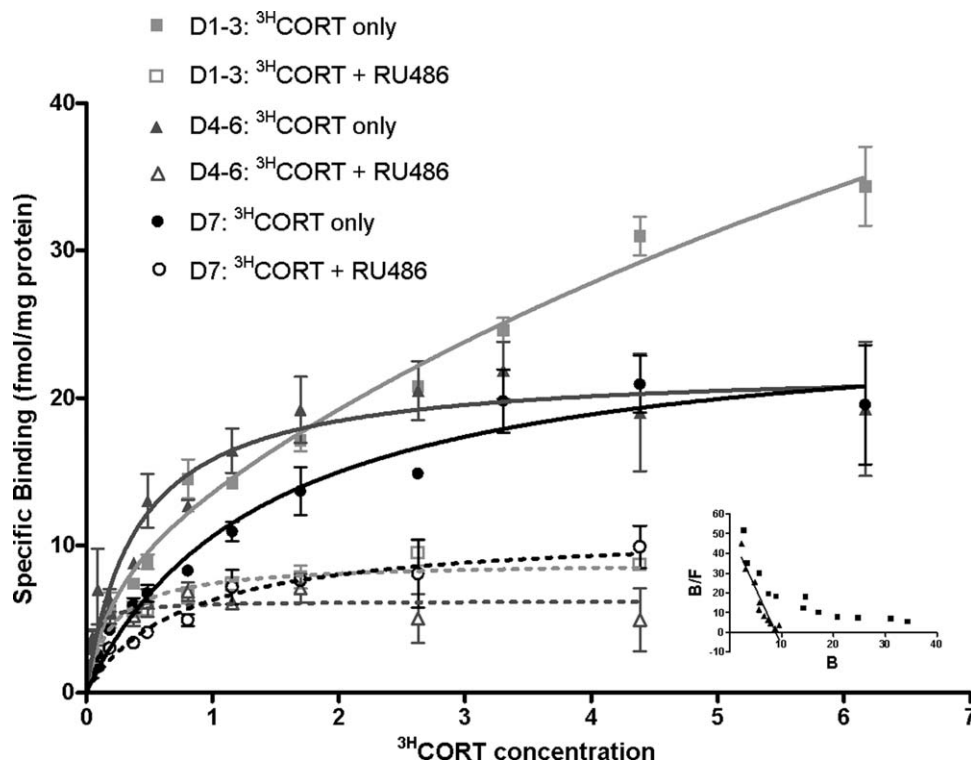


Figure 1 Equilibrium saturation binding of corticosteroid intracellular receptors in white-crowned sparrow (*Zonotrichia leucophrys nuttalli*) nestling brain at different ages. Data shown represent specific binding of ^3H CORT with (open-MR binding) or without (filled-MR + GR binding) 100 nM RU486. Inset is the Scatchard-Rosenthal replot of D1–3 data to illustrate the single binding site of the MR curves and the two sites present in the MR + GR curves.

significantly with age ($F = 5.65$, $p = 0.009$; Fig. 4) where cerebellum of D4–6 or D7 nestlings had significantly lower MR capacity than that of D1–3 nestlings ($p \leq 0.05$). There was a significant sex difference in hindbrain MR ($F = 4.27$, $p = 0.048$) and marginal difference in hippocampus/HVC GR capacities ($F = 3.73$, $p = 0.067$). Female nestlings had higher hindbrain MR capacity and a trend toward lower GR capacity in hippocampus/HVC.

DISCUSSION

White-crowned sparrows show a period of hyporesponsiveness to handling stress early in nestling development (Wada et al., 2007). We hypothesized that this reduced response is partly due to elevated negative feedback resulting from an upregulation of corticosteroid receptors in hypothalamus and hippocampus, the major sites for negative feedback and tonic inhibition in adults (Sapolsky et al., 1984; Herman et al., 1989; Sapolsky et al., 1991). Supporting our prediction, whole brain MR binding capacity decreased with age, indicating a higher negative feedback in newly hatched

chicks compared to ones near fledging. However, this overall decline in MR capacity was driven entirely by a decline in cerebellar MR. No age-related changes were observed in hippocampal or hypothalamic areas, where negative feedback occurs.

In mammals, age-related changes in corticosteroid receptors capacities are often reflected in CORT secretion. Relatively high receptor levels in the brain translate into greater negative feedback and greater cellular response to CORT. A decline in corticosteroid receptors mainly in hippocampus leads to a dampened negative feedback thus an elevation of CORT in aging rats (Sapolsky et al., 1983; Meaney et al., 1988; Peiffer et al., 1991). During rats' SHRP, hippocampal GR is relatively low compared to adults. However, due to low levels of corticosteroid binding globulins and heightened sensitivity of receptors to CORT in young, receptor occupancy is similar between neonates and adults, keeping the CORT secretion low (Viau et al., 1996). In fact, plasma CORT and ACTH levels soar when GR is pharmacologically blocked (Schmidt et al., 2005). Furthermore, brief neonatal handling in rats increases GR in hippocampus permanently (Caldji et al., 2001; Meaney,

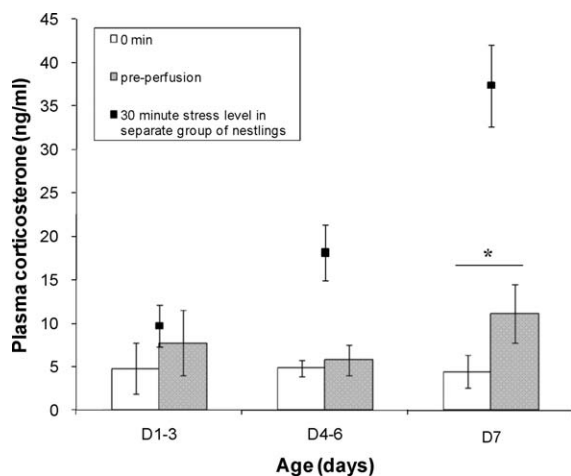


Figure 2 Baseline (<4 min) and preperfusion levels of corticosterone in white-crowned sparrow (*Zonotrichia leucophrys nuttalli*) nestlings used in the whole-brain analysis. Stress-induced levels of corticosterone from the previous study were added for comparison (Wada et al., 2007). * $p \leq 0.05$.

2001). This increase is thought to be responsible for lower adrenocortical response to stress in adults, due to an enhanced negative feedback and tonic inhibition. At the same time, relatively high receptor levels during development may increase vulnerability to excess CORT in certain areas of the brain (Benesova and Pavlik, 1989; Ferguson and Holson, 1999).

Our results in white-crowned sparrows differ from those of mammalian ontogenetic studies in that: (1) it was MR, not GR that changed with age, (2) MR levels declined over time, and (3) this decline in MR capacity was most evident in the cerebellum. We do not know whether receptors in cerebellum are

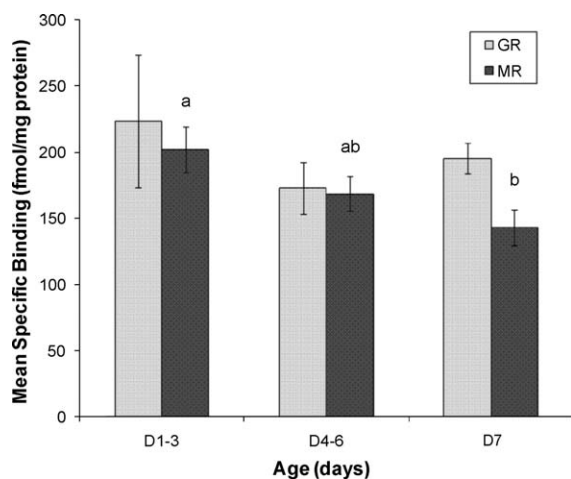


Figure 3 WHOLE BRAIN: Changes in whole-brain GR and MR specific binding in relation to age in white-crowned sparrow (*Zonotrichia leucophrys nuttalli*) nestling brain. Different letters indicate a significance level of 0.05 between age groups.

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Table 2 The Effects of Age and Sex on Corticosteroid Receptors in Five Regions of White-Crowned Sparrow (*Zonotrichia leucophrys nuttalli*) Nestling Brain

		Age		Sex	
		F	p	F	p
Optic lobe	GR	1.46	0.250	0.53	0.472
	MR	1.88	0.172	1.82	0.188
Cerebellum	GR	0.13	0.882	0.13	0.725
	MR	5.65	0.009	0.07	0.788
Hindbrain	GR	0.86	0.434	1.73	0.199
	MR	0.87	0.430	4.27	0.048
Diencephalon	GR	0.54	0.591	0.21	0.648
	MR	0.33	0.725	0.83	0.371
Hippocampus/HVC	GR	0.36	0.702	3.73	0.067
	MR	0.23	0.799	1.09	0.309

Significant effects are in bold. The significant age effect on the cerebellum is shown in Figure 4.

involved in negative feedback. If not, then why does cerebellar MR decline with age?

One possibility is that the high MR levels in early-stage nestling cerebellum are due to a rapid development of the area, as CORT is involved in regulating cellular differentiation and maturation (De Kloet, 1991; Trejo et al., 1995, 2000). In white-crowned sparrows, the cerebellum undergoes the most dramatic morphological development during the nestling period compared to the optic lobe, hindbrain, or diencephalon (see Fig. 5). However, this also leaves the cerebellum more vulnerable to excess CORT. In mammals, the cerebellum also undergoes substantial development during the postnatal period (Bell et al., 1986; Rodier,

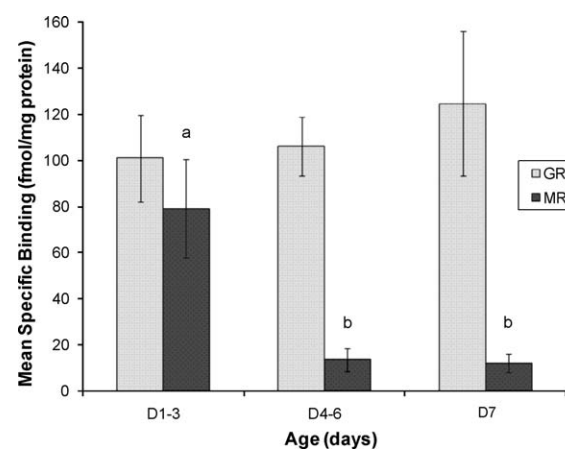


Figure 4 CEREBELLUM: Changes in cerebellar GR and MR specific binding in relation to age in white-crowned sparrow (*Zonotrichia leucophrys nuttalli*) nestling brain. Different letters indicate a significance level of 0.05 between age groups.

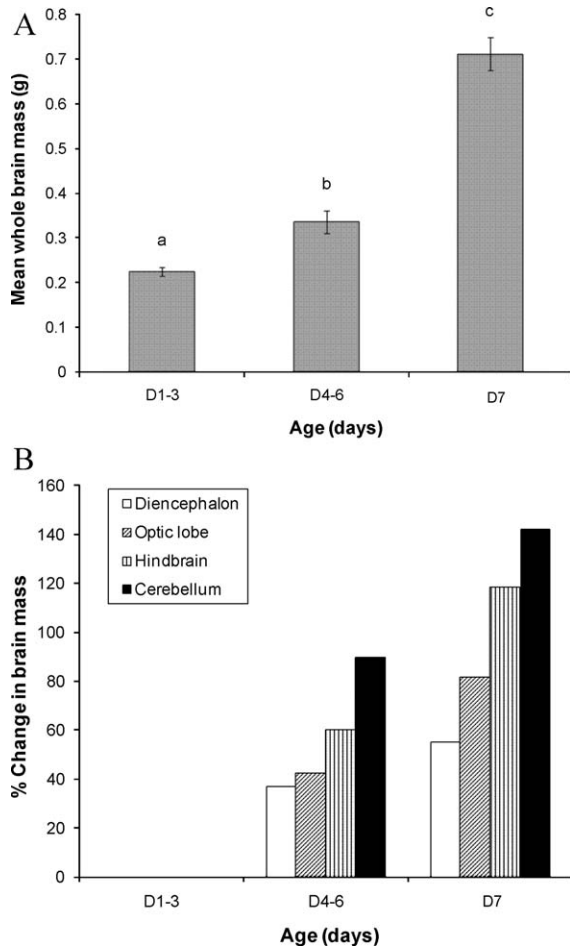


Figure 5 Changes in brain mass in relation to age in white-crowned sparrow (*Zonotrichia leucophrys nuttalli*) nestlings. Figure A represents a change in whole brain mass. Age had a significant effect on whole brain mass ($F = 40.75, p < 0.001$). Different letters indicate significant differences between groups ($p < 0.05$). Figure B represents a percent change in different regions of the brain compared to D1–3.

1988), and contains the highest levels of GR compared with other regions of the brain (Pavlik and Buresova, 1984). As a result, dexamethasone (Dex) administration to rats during this rapid cerebellum growth suppresses protein content in cerebellum, along with a reduction in body weight and whole and regional brain weight (Benesova and Pavlik, 1989; Ferguson and Holson, 1999; Kanagawa et al., 2006). Although other regions of the brain recovered after Dex treatment, the cerebellum retained its deficiency in weight and protein content, again indicating its high sensitivity to glucocorticoids. It is worth noting that in white-crowned sparrows, MR levels are reduced by the time the HPA axis of nestlings is fully functional (middle to late-stage nestlings), (Wada et al., 2007). This means that in optimal conditions CORT levels should not become

detrimentally elevated when cerebellum is most sensitive to CORT.

Another possibility for the observed decline in cerebellar MR levels is the ontogenetic change in morphology of the cerebellum. Mammalian cerebellum compartmentalizes into parasagittal stripes during development (Larouche and Hawkes, 2006). During this time, the thickness of granule and Purkinje cell layers changes. In rats and ferrets (*Mustela Putorius furo*), the relative size of granule layer in cerebellar cortex decreases with age, from postnatal days 28 to 56 (Christensson et al., 2007). If avian cerebellum undergoes a similar ontogenetic compartmentalization and has more MR in the granule layer than the rest of cerebellum, then MR levels would decline with age. Further studies examining the ontogenetic change in MR distribution in avian brain are needed to confirm this possibility.

Alternatively, it is possible that endogenous elevation in CORT masked the true MR levels in the late-stage nestlings. Mitotane treatment successfully suppressed CORT levels in the first two age groups, while the oldest age group retained a slight but significant increase in CORT prior to perfusion. However, such a scenario is unlikely because there was no correlation between preperfusion CORT and MR levels, and no age difference in preperfusion levels of CORT. Additionally, MR levels were the same between the middle- and late-stage nestlings, while endogenous CORT was only elevated in the late-stage nestlings. And lastly, if endogenous CORT levels were masking MR in the older nestlings, MR levels would have declined in every brain area, not just the cerebellum.

In conclusion, our findings suggest that the ontogenetic changes in GR and MR in the brain of white-crowned sparrow nestlings do not contribute to the attenuated adrenocortical responses during the early post-hatch period. Little is known about the role of corticosteroid receptors in cerebellum in young birds and more studies are needed to determine the significance of the ontogenetic decline in MR in cerebellum.

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